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CCR6-dependent positioning of memory B cells is essential for their ability to mount a recall response to antigen

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Abstract

Chemokine-dependent localization of specific B cell subsets within the immune microarchitecture is essential to insure successful cognate interactions. While cognate interactions between T cells and memory B cells (B_{mem})⁵ are essential for the secondary humoral immune responses, the chemokine response patterns of B_{mem} cells are largely unknown. In contrast to naïve B cells, this study shows that antigen-specific B_{mem} cells have heightened expression of CCR6 and a selective chemotactic response to the CCR6 ligand, CCL20. While CCR6 appears to be non-essential for the initial clonal expansion and maintenance of B_{mem} , CCR6 is essential for the ability of B_{mem} to respond to a recall response to their cognate antigen. This dependency was deemed intrinsic by studies in CCR6-deficient mice and in bone-marrow chimeric mice where CCR6 deficiency was limited to the B cell lineage. Finally, the mis-positioning of CCR6-deficient B_{mem} was revealed by immunohistological analysis with an altered distribution of CCR6-deficient B_{mem} from the marginal and perifollicular to the follicular/germinal center area.

⁵Abbreviations: B_{mem} , memory B cell; BM, bone marrow; PC, Plasma Cell;

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Introduction

Long-lived serological memory is the product of long-lived memory B cells (B_{mem}) and plasma cells (PC). B_{mem} cells are long-lived although their half-life is as-yet undefined in both mouse and man (1). B_{mem} cells are quiescent, they can be IgM memory or express isotype-switched and somatically hypermutated membrane immunoglobulin (2, 3). IgM B_{mem} cells are produced independent of T cell help, differentiate in the absence of GC structure (4), produce natural antibodies (3) and part of circulating marginal zone B cells (2). Upon exposure to their cognate antigen, Ig switched and IgM⁺ B_{mem} , rapidly divide to daughter B_{mem} and differentiate to PC (5). Lastly, PCs residing in the bone marrow (BM) are long-lived cells, quiescent, terminally-differentiated and produce Ig for long periods of time (6).

The appropriate anatomic localization of individual B cell subsets is essential to execute their specific functions, with chemokines controlling B cell localization within the immune microarchitecture (7). For example, CXCR5 and CCR7 orchestrate the precise localization of naïve B cell in secondary lymphoid organs and spleen (8–10). In addition, it has been demonstrated that the differential expression of CXCR4 and CXCR5 plays an important role in the localization of antigen-activated B cells during germinal center (GC) formation, permitting the selection of the most suitable clones during PC differentiation (11). Finally, downregulation of CXCR5 and CCR7 expression by PCs with sustained expression of CXCR4, mediates the migration of PCs from secondary lymphoid organs to the BM and sustains PC survival (12, 13).

CCR6 is expressed in different subsets of CD4 and CD8 T cells (14), immature DCs (15), NK T cells (16) and B cells (17, 18). Prior work demonstrated that B_{mem} cells also express CCR6, and it was proposed that CCR6 may contribute to the migration of this population to the mucosal tissue (19). However the role of this receptor in secondary humoral immune responses was not studied.

The studies presented here show that antigen-specific B_{mem} cells express heightened levels of CCR6 and display an increased chemotactic response to the CCR6 ligand, CCL20, when compared to naïve B cells. Neither the primary humoral response nor the initial generation and maintenance of antigen-specific B_{mem} cell are impaired in CCR6-deficient mice. However, genetic deletion of CCR6 in B cells prevents antigen-specific B_{mem} from mounting an effective secondary response upon antigen re-challenge and disrupts their normal CCL20-dependent anatomic distribution in the spleen. Together these observations show that CCR6 is essential for appropriate anatomical positioning of B_{mem} and the ability of B_{mem} to be recalled to their cognate antigen.

Material and Methods

Mice and Immunizations

These studies were approved and conducted in accredited facilities in accordance with the Institutional Animal Care and Use Committee (IACUC) of Dartmouth College (Lebanon, NH, USA) and UK Animals (Scientific Procedures) Act 1986 (Home Office license number

PPL 70/7102). C γ 1-Cre mice were provided by K. Rajewsky (Harvard Medical School, Boston MA) and S. Casola (Fondazione Italiana per la Ricerca sul Cancro Institute of Molecular Oncology Foundation, Milan, Italy). C57/BL6 mice were purchased from the NCI and Charles River. CCR6^{-/-}, Rosa YFP^{fl/fl} and μ MT mice were purchased from The Jackson Laboratory. All animals were maintained in a pathogen-free facility at Dartmouth Medical School and King's College London. For primary immunizations, 10 μ g of PE (Chromoprobe) adsorbed to prepared alum was injected intraperitoneally (i.p.) in a volume of 200 μ l. For secondary challenge, 10 μ g of PE in PBS in a volume of 200 μ l was injected i.p.

Cell Preparation

To sort memory B cells, single cell suspensions of lymphocytes were prepared as previously described (1). Splenocytes were incubated with anti-IgD-biotin, anti-IgM-biotin, anti-CD8 α -biotin and anti-CD4-biotin, with these cells removed using the EasySep biotin Selection kit for mouse cells (Stem Cell Technologies).

Flow Cytometry

Antibodies against the following antigens were used: B220 (clone 6B2), IgG1 (clone A85-1), IgD clone (11-26c), IgM (clone II/41), CD4 (clone GK1.5), CD8 (clone 2.43), CD38 (clone 90), CD23 (clone B3B4) and CCR6 (clone 29-2L17), Sign-R1 (clone ERTR9). PE-binding cells were detected by staining with 1 μ g/ml PE. Samples were acquired with a refurbished Becton Dickinson FACSCAN running CellQuest software (BD Bioscience) and data were analyzed by FlowJo (Tree Star) software. Cell sorts were performed on a BD FACSaria with 10,000–100,000 cells sorted and postsort analysis indicating purities exceeding 98% (Flow Cytometry Facility at Dartmouth Medical School).

Real Time PCR

Total RNA was isolated from either purified memory or naïve B cells using TRIzol (Invitrogen) followed by secondary purification over RNeasy columns (QIAGEN) with a DNase-I treatment step. 1 μ g of DNA-free RNA was reverse transcribed to cDNA using Omniscript RT (QIAGEN). Real-time PCR was performed with the SYBR Green PCR Core Kit (Applied Bio- systems) on an iCycler iQ instrument (Bio-Rad Laboratories). Amplification conditions were 95°C for 8 min, followed by 40 cycles of 94°C for 15 s, 62°C for 45 s, and 72°C for 15 s. Primers for the control gene mouse β -actin were as follows: forward, 5'-CCAATGTGTCCATGTCATTT-3' and reverse, 5'-CAATA-GTGATGACCTGGCCGT-3'. CCR6^{-/-} used was as follows: forward, 5'-CCCAGCACATCATAGCATTG-3' and reverse, 5'-TGGGAGAGCAGAGGTGAAGT-3'. For CCL20 gene expression, TaqMan gene expression assays containing FAM dye-labeled TaqMan MGB probe was used for mouse CCL20 (Mm01268754_m1) in multiplex with primer-limited assays for β -actin endogenous control, containing VIC/MGB probes. Real-time quantification was performed using TaqMan gene expression master mix on a Bio-Rad CFX96 optical reaction module on a C1000 thermal cycler. Data were analyzed using CFX Manager Software (Bio-Rad). Relative RNA expression was determined using the formula $\text{relative expression} = 2^{-(CT - CT_{\beta\text{-actin}})} \times 1,000$, where $CT = (\text{cycle threshold [CT] gene of interest} - CT_{\beta\text{-actin in experimental sample}}) - (CT_{\text{gene of interest}} - CT_{\beta\text{-actin in a no-}})$

template control sample) (see the CT method, Taqman® Bulletin 2: Applied Biosystems).

Chemotaxis assays

Chemotactic migration assays were performed, as previously described (15). Briefly, between 5×10^6 splenocytes were placed in the upper chamber of 5- μ m of 24-well plates transwell inserts (Corning Costar, Cambridge, MA), in contact with 600 μ l of medium (RPMI-1640 with 10% FBS) with or without chemokine. After 2 h, a fixed volume of counting beads (Polysciences Inc., Warrington, Pennsylvania, USA) was added to each well for normalization, and the specific migration of B_{mem} or naïve B cells was enumerated by flow cytometry after staining for the B lymphocyte subsets of interest. Mouse RANTES, CCL17, S1P, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CCL20, CCL21 and CCL22 were purchased from Peprotech Inc.

Microscopy/Immunofluorescence

For germinal center structure analysis, tissues were snap-frozen and 8 μ m sections were fixed with methanol prior to staining with PNA-FITC, anti mouse/human B220-A647 and anti mouse monoclonal antibody ERTR9-biotin plus streptavidin-A455. Slides were analyzed by a confocal microscope (LSM510Meta, Zeiss).

To detect B_{mem} and YFP expression, tissue was removed, fixed in 4% paraformaldehyde, followed by overnight saturation in 10% sucrose at 4°C. Tissue was then snap frozen in OCT Tissue-Tek freezing medium (Sakura). Cryostat sections of 7 μ m were air dried and washed in PBS. Blocking was performed with 20% normal horse serum (PAA Laboratories Inc.) for 15 minutes at room temperature. Sections were incubated with Alexa Fluor 488 - conjugated anti-GFP (Life Technologies) and biotin-conjugated anti-mouse B220 (Clone RA3-6B2, Ebioscience), anti-mouse IgM/IgD (clone 11/41; 11-26c, Ebioscience), or anti-mouse MOMA (Abcam), followed by streptavidin Alexa Fluor 594 (Life Technologies). Nuclei were visualised by staining with 1 μ g/ml DAPI (Invitrogen). Negative controls were Rosa YFP mice (no GFP) or stained with isotype-matched antibodies. Images were acquired on an Olympus BX51 microscope using Micro-Manager software (Vale Laboratory).

ELISPOT/ELISA Analysis

ELISPOTs were developed as previously described (1), single cell suspensions from bone marrow and spleen were counted and were apportioned to PE coated Multiscreen 96-well plates (Millipore) with twofold serial dilutions made before incubation for 4h at 37°C. PCs were detected by HRP-conjugated anti-mouse IgG1 polyclonal antibody (Southern Biotechnology Associates, Inc.).

For ELISA analysis, plates were coated with 10 μ g/ml PE overnight in PBS, blocked with PBS + 5% FBS, washed, and then 1:2000 diluted serum added at serial 1:2 dilutions. Serum from either a PE hyper-immunized mouse was included on each plate as a reference between plates and between experiments and used to generate a standard curve, with these serum allotted the value 8000 arbitrary units. Antibody levels were detected with IgG-AP

(Southern Biotechnology) and developed with 1mg/ml pNPP (Sigma) in 0.05mM sodium carbonate buffer.

Irradiation and Chimera Generation

For the generation of mixed chimera C57BL6 mice were lethally irradiated (600 rad) twice and reconstituted (i.v.) with a mixture of 5×10^6 bone marrow cells of 80% μ MT origin and 20% with wt or CCR6KO cells. Successful grafting was determined by flow cytometry of peripheral blood 8 weeks after transplantation. Immunizations and re-challenge responses were performed 8 weeks and 2 month after bone marrow transplant respectively.

Statistics

Results are expressed as mean plus or minus SEM. Two-tailed Student *t* test with unequal variance was used to evaluate the statistical significance of the data.

Results

Increased expression of CCR6 and enhanced chemotaxis to the CCR6 ligand, CCL20, by memory B cells

Using Phycoerythrin (PE) as an immunization antigen, the emergence and differentiation of PE-binding B_{mem} *in vivo* was quantified, as we have previously published (1, 20). We have previously shown (1) that PE-binding B_{mem} cells were defined as $B220^+PE^+CD38^+IgD^-IgM^-IgG1^+$ and naïve B cells as $B220^+IgM^+CD38^+IgD^-IgG1^-PE^-$ (Supplementary Fig. 1A). B_{mem} and naïve B cell CCR6 mRNA and protein expression levels were determined in C57/BL6 mice immunized with PE 60 days previously. PE-specific B_{mem} and naïve B cells were sorted with a purity over 95% and *Ccr6* transcript levels were assessed. An increased level of *Ccr6* transcript in PE-binding B_{mem} cells was observed when compared to that observed in naïve B cells (Fig. 1A). Flow cytofluorimetric analysis corroborated the heightened expression of CCR6 in B_{mem} by showing a higher percentage of B_{mem} expressed membrane CCR6 compared to naïve B cells (Fig. 1, B and C). Moreover, B_{mem} have a higher MFI in CCR6 expression when compared to naïve B cells (Fig. 1B). CCR6 expression was also analyzed in immature B cells ($B220^+IgM^{hi}IgD^-$), mature B cells ($B220^+IgM^{int}IgD^+$), pre B cells ($B220^+IgM^-IgD^-$) and PCs ($B220^-CD138^+IgD^-$) from BM (21). Mature B cells expressed similar levels of CCR6 compared to naïve B cells and CCR6 expression is increased during the development of B cells (Fig. 1, D and E). Neither pre B cells nor PCs express this chemokine receptor (Fig. 1, D and E). These results together establish that B_{mem} cells express increased levels of CCR6 mRNA and protein when compared to other B cell subsets, including PCs.

To evaluate whether CCR6 expression correlated with a heightened chemotactic response to CCR6 ligand, CCL20, *in vitro* migration assays were performed. Splenic B cell subsets were evaluated for their *in vitro* capability to migrate to different concentrations of a spectrum of chemokines. *In vitro* migration assays showed (Fig. 1, F and Supplementary Fig. 1B and C) that B_{mem} preferentially migrated in response to CCL20, in a dose-dependent manner when compared to naïve B cells. B_{mem} , like naïve B cells were also able to migrate to CXCL12 and CXCL13. In addition, although naïve B cells also express CCR6, they did not migrate in

response to CCL20 (Supplementary Fig. 1B). These results established that B_{mem}, and not naïve B cells are able to migrate in response to CCL20.

CCR6 is dispensable for the primary humoral immune response

The expression of CCR6 by naïve B cells potentially implicated CCR6 as playing a role in the primary humoral immune response (Fig. 1B). To evaluate the role of CCR6 in a primary B cell response, the ability of CCR6-deficient mice to mount a T-dependent primary antibody response was evaluated. The lack of CCR6 did not disrupt splenic architecture in PE-immune mice and there was no difference in the GC structure (Fig. 2A) or frequency of GC B cells, as defined by B220⁺CD95⁺GL-7⁺CD38⁻ B cells (Fig. 2, B, C, D and E) 14 days after immunization. Furthermore, there was no impact of CCR6-deficiency on anti-PE serum antibody titers or PE⁻ specific IgG1⁺ BM-ASC at day 14 and 28 after immunization (Fig. 2F and 2G). These data show that CCR6 expression is not required for GC formation, the primary generation of antigen-specific ASC or enhanced serum antigen-specific antibody titers following primary immunization.

The heightened expression of CCR6 on B_{mem} suggested that it might play a role in the initial differentiation of B_{mem} during the primary response. To this end, CCR6 deficient and wild type mice were immunized with PE and after 4 months, the percentage of B_{mem} was analyzed by flow cytometry. B_{mem} were defined as B220⁺PE⁺CD38⁺IgD⁻IgM⁻IgG1⁺, as shown in Figure 2H, the frequency of B_{mem} cell in CCR6^{-/-} mice and WT mice were indistinguishable between lymph nodes and spleen. These data established that CCR6 was dispensable for the differentiation of B_{mem} during the primary immune response.

Involvement of CCR6 in the recall response of B_{mem}

CCR6 is not involved in the development of the primary humoral immune response nor in the early differentiation of antigen-specific B_{mem} or ASC. Additional studies were designed to address the potential role of CCR6 in B_{mem} maintenance and recall responsiveness. As such, we analyzed the recall response in immune WT and CCR6-deficient mice. Immune CCR6-deficient and WT mice immunized 120 days previously were re-challenged with PE and 5 days later, the number of antibody secreting cells (ASCs) was quantified. The results show that there was a marked reduction in the number of PE-specific IgG1 ASCs in both spleen and BM of CCR6-deficient mice compare to WT mice (Fig. 3A and B). Also observed was a significant reduction in the serum titers of anti-PE IgG1 (Fig. 3C). These observations indicated that the recall capacity of B_{mem} is impaired in the absence of CCR6.

To determine whether the defect in B_{mem} function in CCR6-deficient mice is intrinsic to the B cell lineage, mixed BM chimeras were produced. Reconstitution of irradiated wild-type hosts with a mixture of μ MT BM with either CCR6-deficient or WT BM resulted in chimeras in which the B cell compartment was derived from CCR6-deficient or WT BM precursors and all other compartments were predominantly of a WT genotype (Fig. 3D). After the reconstitution of the hematopoietic compartment, the mice that were immunized 60 days previously, were re-challenged with PE and 5 days later, the number of ASCs was analyzed by ELISPOT. The data showed that in both spleen and BM there were marked reductions in the number of ASCs in the chimeras where the B cells were derived from

CCR6-deficient BM compared to those chimeras where the B cells were derived from WT BM (Fig. 3E and F). These results show that B lineage-restricted expression of CCR6 is critical for B_{mem} cells to respond to a secondary challenge by antigen.

Involvement of CCR6 in the localization of B_{mem}

The immunohistological tracking of B_{mem} is challenging given the lack of a definitive set of easily usable markers to identify them *in situ* (22–24). Nonetheless, it was important to determine whether the deficiency of CCR6 resulted in the altered anatomic localization of B_{mem}. As such, the distribution of B cells in WT and CCR6-deficient mice was assessed. Tracking of B_{mem} was afforded by analysis using a mouse whereby Cre recombinase was inserted in the Cγ1 locus interbred with a mouse engineered with a conditional Rosa26-EYFP allele (called here, RosaYFP) (25). In this strain, every post-GC B cell, including B_{mem}, expresses YFP. Flow cytofluorimetric analysis reveals that less than 1% of total B cells express YFP⁺ in the steady state (data not shown) (25). Subset analyses of the YFP⁺ cells in immune RosaYFP mice showed that the YFP⁺ population is composed of IgM/IgD⁺ and IgG⁺ activated B cells (Fig. 4A). Based on flow cytofluorimetric analysis, it was not evident that the YFP⁺ B cells that were IgM/IgD⁺ were B_{mem}, and as such were independently scored for their distribution. The splenic localization of YFP⁺, IgM[−]/IgD[−], IgG1⁺ were quantified as a measure of B_{mem} distribution (Fig. 4B and F).

First, no difference was found in the percentage of activated or polyclonal B_{mem} cells in either the Cγ1-cre x RosaYFP x CCR6^{−/−} and Cγ1-cre x RosaYFP mice (Fig. 4A and B), as expected from our analysis of the frequency of B_{mem} in CCR6^{−/−} mice. Second, staining for YFP⁺ cells in WT mice revealed that the majority of the B220⁺, YFP⁺ cells were distributed widely outside of follicular areas. Note that double positive cells (B220⁺, YFP⁺) did not appear yellow because the YFP stain and the B220 stain were localized to the same cells but one stain was cytoplasmic (YFP) and the other localized to the plasma membrane compartment (B220). Third, polyclonal B_{mem} that were enumerated in RosaYFP mice by loss of IgD and IgM, were equally located outside the follicle/GC areas of the spleen, as predicted by prior studies showing the capacity of B_{mem} to localize to the marginal zone (Supplementary Fig. 2A) (24).

Finally, in the CCR6-deficient mice, where there was over a 6-fold increase of B_{mem} cells found in follicular areas compared to what was observed in WT mice (Fig. 4C, D, F). The results show that in the spleen of Cγ1-cre x RosaYFP x CCR6^{−/−} mice, B_{mem} are redistributed to follicular/GC structures, which are associated with CD35 expression and PNA (Fig. G and H, supplemental Fig. 2E), whereas in control mice RosaYFP B_{mem} equally localize between perifollicular and marginal zone areas, co-localizing with MOMA expression (Supplementary Fig. 2A and B).

The striking increase in follicular redistribution of virtually all YFP⁺ B cells in the CCR6^{−/−} mice raised a number of additional questions. To determine whether an increase in GC numbers in the spleen of Cγ1-cre x RosaYFP x CCR6^{−/−} in steady state could account for the redistribution of YFP⁺ cells, GC numbers were quantified. Although in CCR6^{−/−} mice there is a tendency towards a higher percentage of GC B cells, this difference is not statistically significant in both percentage and absolute number (Fig. 4I and Supplementary

Fig. 2D and E), suggesting that the accumulation of activated B cells in the follicular areas is not due to an increase in GC B cells. Peri-follicular redistribution could also be the result of heightened expression of CXCR5 on B_{mem} relative to WT B_{mem}, and as such, the expression of CXCR5 was analyzed. CCR6^{-/-} and WT mice express identical densities of CXCR5 (Supplementary Fig. 3). This indicates that the selective migration of CCR6^{-/-} B_{mem} is not due to heightened CXCR5 expression. Therefore, at this time, it is not clear why the loss of CCR6 results in the redistribution of B cells.

CCL20 is the ligand for CCR6 and as such, CCL20 gene expression in different splenic population of immune cells and whole spleen was evaluated in WT and CCR6^{-/-} mice. qPCR for CCL20 gene indicated that CD4⁺ T cells were the major producers of CCL20 in the spleen (Fig. 4J). Our results also show that the CCL20 abundance in total spleen is similar that found in CD4⁺ T cells, indicating that the CCL20 signal is truly enriched in CD4⁺ T cells. Taken together, these results suggest that the lack of CCR6 expression on activated B cells prevents co-localization of B_{mem} with CCL20-expressing CD4⁺ T cell in the spleen, impacting on their ability to respond to antigen.

Discussion

Chemokine receptors play crucial role for the appropriate localization of B_{mem} in secondary lymphoid organs and impact on the ability of B_{mem} to mount an effective secondary immune response. To our knowledge, the findings presented here are the first to establish a critical role of CCR6 in B_{mem} function. The data shows that a high frequency of B_{mem} cells express CCR6 and have a heightened chemotactic response to CCL20 compared to naïve B cells. It is known that there is broad CCR6 expression across B cell subsets in the mouse and CCR6 is expressed at higher levels transiently during cognate activation of B cells (26, 27). CCR6-deficient mice have a faster GC response compared with littermate controls, resulting in an increase of low affinity antigen-specific plasmablasts (26). Interestingly though, like in this report, prior authors showed that there was heightened histological accumulation of activated B cells in the GC/follicle in the CCR6^{-/-} mice. In studying the primary response to PE, we did not observe any difference in the primary immune response, with regard to GC formation, BM-PCs or antibody titers when compared to control mice. Our findings show that B_{mem} retain high expression of CCR6 during the immunization period compared with that constitutively expressed on naïve B cells. A significant impact of B cell intrinsic CCR6-deficiency was a loss in the secondary recall response of B_{mem}. Because chemokines are important in the anatomic migration and localization of cells within secondary lymphoid organs (28), it is proposed that the absence of CCR6 expression impacts on the localization of established B_{mem} to receive the suitable signals to become PCs. Alternatively, CCR6 may play a role in PC survival, however, this unlikely since our own data (Figure 1E) and published studies report that PCs do not express CCR6 (29).

Loss of CCR6 appears to dramatically alter the histological distribution of activated B cells. Attempts to detect B_{mem} *in vivo* in order to identify their location and migratory patterns have been met with limited success (22–24). It has been reported that there is preferential localization of B_{mem} adjacent to contracted GCs in the spleen, which may be chemokine dependent, and which is different to the behavior of primary B cells that are responding to

antigen (22). Furthermore, it has been argued that B_{mem} cells localized in marginal zone area are IgM-B_{mem} cells (22–24). In our model, using YFP expression under C γ 1-Cre promoter to help identify B_{mem}, it was observed that polyclonal B_{mem} cells are widely distributed across the marginal zone and perfollicular area, suggesting that it is not the exclusive location for antigen experienced IgM⁺ B cells as was proposed previously (22–24). Most importantly, we observed an accumulation of B_{mem} in the follicular areas in the absence of CCR6 expression. Why this redistribution of B_{mem} impairs their ability to mount a recall response to the cognate antigen is currently under investigation.

qPCR analysis in immune mice revealed that CCL20 is expressed on CD4 T cells in the spleen, and provides a reasonable basis for suggesting that antigen-primed CD4⁺ T cell produce CCL20, and instruct the chemotaxis of CCR6-bearing B_{mem} to facilitate cognate interactions in the secondary humoral response. CCL20 is expressed at low levels in splenic CD4⁺ T cells at steady state compared with Peyer's patches. However, it is significantly induced by pro-inflammatory signals via TLRs agonists or TNF α (30). CCL20 is not the only ligand for CCR6; β -defensins and other non-chemokine proteins can also be ligands for CCR6. However, their affinity is much lower compared to CCL20 (31). CCR6 expression and the chemotactic response to CCL20 on B_{mem} cells is consistent with CCR6 being an important molecule in the systemic migration B_{mem} to mucosal and effector sites (18, 19), an event perhaps critical in the lifespan of B_{mem}. Loss of CCR6 disorganized the co-localization of CCL20 producing T cells and B_{mem} which likely contributed to the impaired recall responses of B_{mem} to their cognate antigen.

In conclusion, CCR6 is highly expressed on B_{mem}, and has an important role in the B_{mem} function and localization in secondary lymphoid organs. Due to the profound role of CCR6 in the regulation of secondary humoral immune responses, this chemokine is an attractive target in diseases where the recall responses of B_{mem} contribute significantly to disease pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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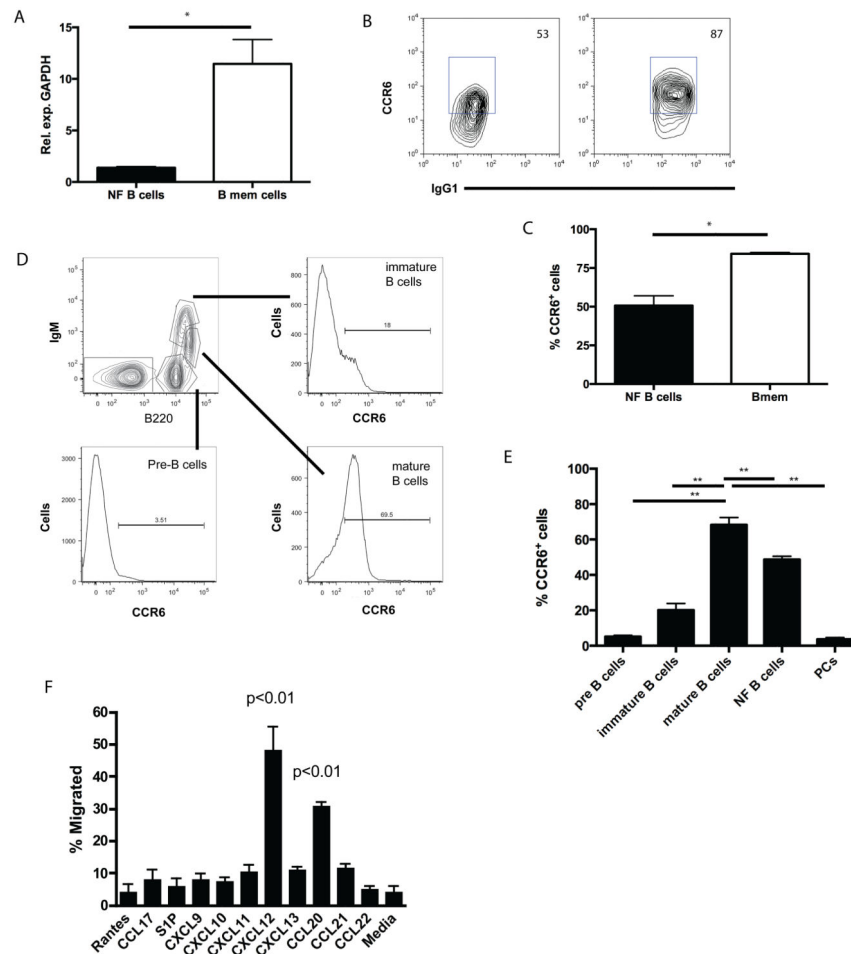


Fig. 1. CCR6 expression on memory B cells and their chemokine response profile

(A) PE-specific B_{mem} cells (B220⁺ CD38⁺ PE⁺ IgG1⁺ and IgM/IgD/CD4/CD8⁻) and naïve B cells (NF B cells) (B220⁺ CD38⁺ CD23⁺ IgG1⁻ and IgM/IgD⁺) were sorted and mRNA expression levels of CCR6 were analyzed. (B) Flow cytometry analysis of CCR6 expression on naïve B cells (left panel) and B_{mem} cells (right panel). Numbers indicates percentage of CCR6 positive cells. (C) Quantification for CCR6 expression in naïve and B_{mem} cells by flow cytometry in B is shown. (D) Flow cytometry analysis of CCR6 expression on immature B cells (B220⁺ IgM^{hi} IgD⁻), mature B cells (B220⁺ IgM^{int} IgD⁺), pre B cells (B220⁺ IgM⁻ IgD⁻) and PCs (B220⁻ CD138⁺ IgD⁻) from bone marrow. (E) Percentage of CCR6 positive cells of each subset in D and NF B cells is shown. All data are representative of at least three independent experiments with at least 3 mice/group. (F) Quantification of chemokine profile response at 250 ng/mL of B_{mem} cells from 3 independent experiments, each sample in duplicate. *p<0.01, ** p<0.001.

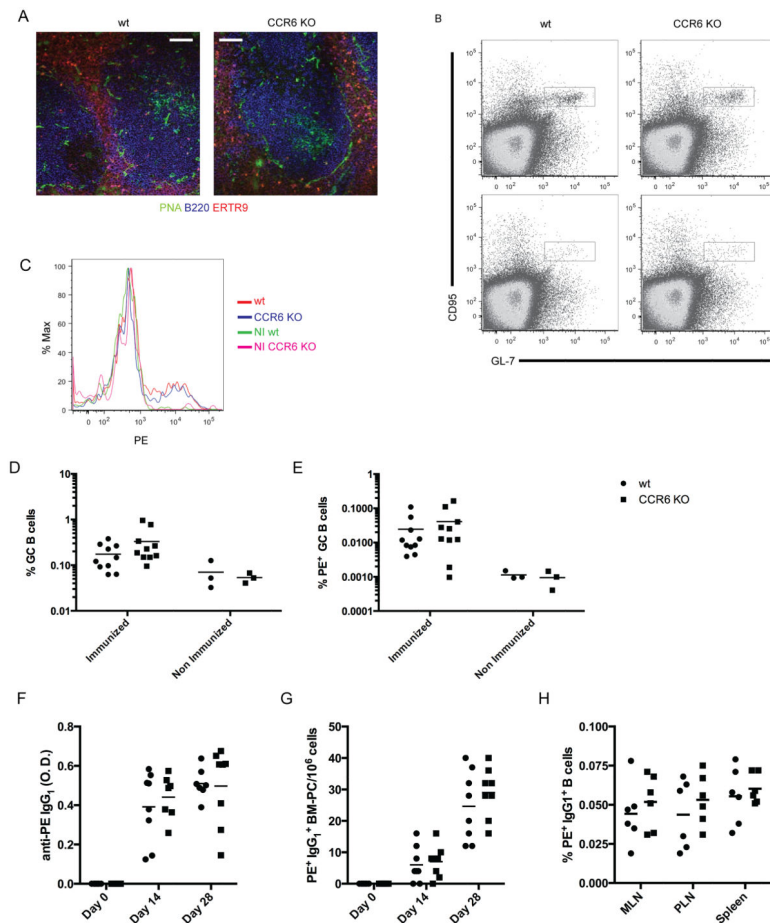


Fig. 2. CCR6 expression on B cells does not affect the primary humoral response or memory B cell development

(A) CCR6KO (right panel) or wt (left panel) mice were immunized with 10 μ g of PE adsorbed in alum, after 7 days, the spleen were frozen and stained with PNA-FITC (Green), B220-alexafluor647 (Blue) and CD4-biotin plus streptoavidin-alexafluor455 (Red) for germinal center analysis. The original magnification is 10x. A representative image of two independent experiments with 3 mice/group. Bar represents 80 μ m. (B) Flow cytometry analysis of GC B cells (B220⁺CD38⁻CD95⁺GL-7⁺) in wt or CCR6KO mice, non-immunized (NI; bottom panels) or immunized (top panels) with PE in alum 14 days before of the analysis. (C) Histogram of PE binding in GC B cells gated in B is shown. (D) Percentage of GC B cells of total B220⁺ cells at day 14 after immunization is shown. (E) Quantification of PE specific GC B cells of total B220⁺ cells is shown. One representative test from two independent experiments with at least 3 mice/group. (F) CCR6KO or wild-type mice were immunized with 10 μ g of PE adsorbed in alum, non-immunized mice (day 0) and 14 and 28 days after immunization, the serums were collected to analyze the production of anti-PE IgG1 titers by ELISA. (G) BMs were collected at the same time point than F to analyze number of PE⁺ IgG1⁺ BM-ASC by ELISPOT. Quantification from two independent experiments with at least 3 mice/group. (H) CCR6KO or wt mice were immunized with 10 μ g of PE in alum. 120 days after, the percentage of PE specific Bmem cells (PE⁺IgG1⁺B220⁺CD38⁺IgM/IgD⁻) were evaluated in mesenteric (MLN), peripheral

lymph (PLN) node and spleen by flow cytometry. The graph shows the quantification of two independent experiments with 3 mice/group.

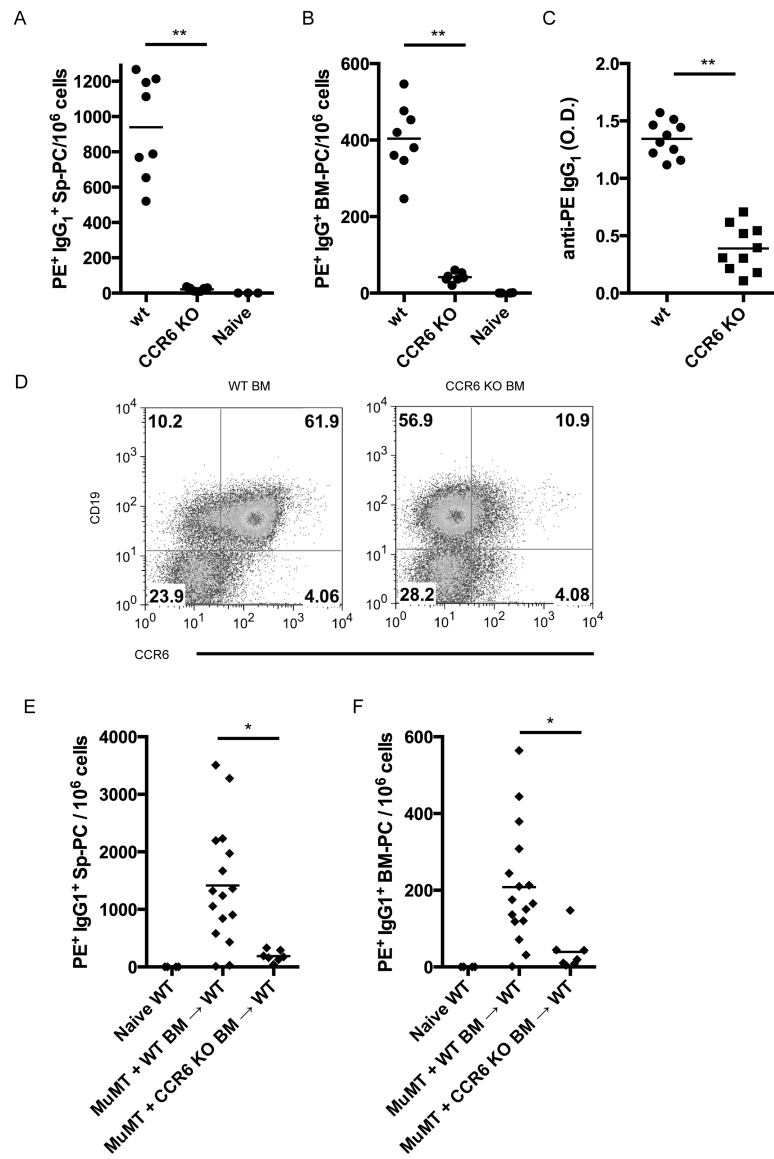


Fig. 3. The secondary humoral response is defective in CCR6^{-/-} mice

CCR6KO and wt mice were immunized with 10 ug of PE in alum, after 120 the mice were re-immunized with 10 ug of soluble antigen PE and 5 days later, the number of anti-PE+ IgG1+ PC from spleen (A) and bone marrow (B) were analyzed by ELISPOT. (C) Quantification of anti-PE IgG1 levels after recall response in serum from (A and B). Quantification of two independent experiments with 4 mice per group. **p<0.001. (D) Representative density plot of CCR6 expression on B cells after the generation of bone marrow chimeras from CCR6KO (right panel) or wild type (left panel) donor-derived. Number of donor-derived of PE-specific IgG1 PC in spleen (E) and bone marrow (F) analyzed by ELISPOT in chimeric CCR6KO and wt mice immunized with 10 ug of PE in alum and challenged 60 days after with 10 μ g of soluble PE. *p<0.01.

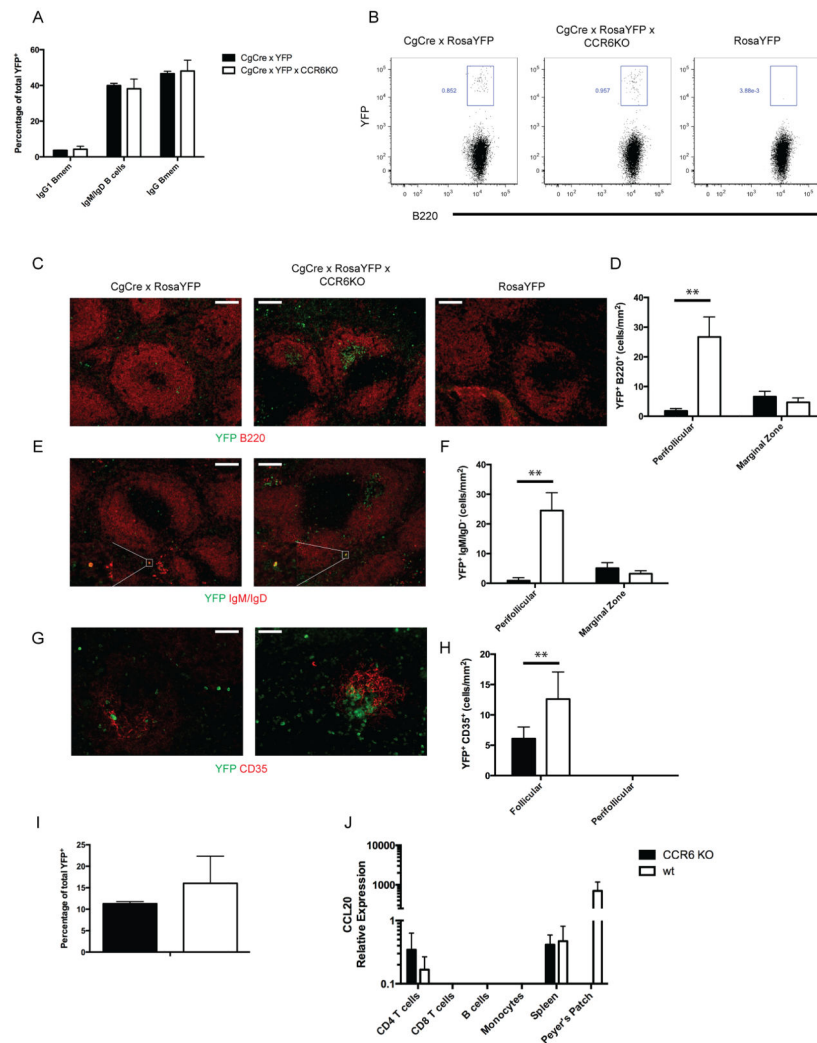


Fig. 4. CCR6 affects the localization of B_{mem} cells

(A) Quantification of splenic YFP⁺ B cell composition from Cγ1-cre x ROSAYFP x CCR6^{-/-} or Cγ1-cre x ROSAYFP is shown. (B) Percentage of polyclonal B_{mem} (B220⁺IgG1/YFP⁺CD38⁺Dump⁻CD80⁺) from spleens of Cγ1-cre x ROSAYFP x CCR6^{-/-} or Cγ1-cre x ROSAYFP is shown. (C, E, G) Histology of YFP expression (green) co-localizing with B220 (C), IgM/IgD (E) and CD35 (G) in red. (D, F, H) Quantification of YFP⁺B220⁺ (D), YFP⁺IgM/IgD⁺ (F), YFP⁺CD35⁺ (H) on histological section of the spleen in the marginal zone and perifollicular area of Cγ1-cre x ROSAYFP x CCR6^{-/-} or Cγ1-cre x ROSAYFP mice. (I) Percentage of GC B cells (CD95⁺GL-7⁺) of total YFP⁺ B220⁺ cells by flow cytometry is shown. (J) CCL20 gene expression on splenic CD4, CD8 T cells, B cells and monocytes, in addition to whole spleen and Peyer's patch tissue. Graph represents 2 independent experiments with 4 mice per group.